Journal of Forestry Research (2010) 21(2): 189–192
DOI 10.1007/s11676-010-0030-2

RESEARCH PAPER

Plasmolysis treatment enhances the expression of callose synthase gene in zygotic embryos of *Eleutherococcus senticosus*

XILIN Hu • AN Yan • XIA De-an • YOU Xiang-ling

Received: 2009-10-09; Accepted: 2009-11-26

Abstract: In previous study we reported that pretreatment with plasmolysis enhanced somatic embryo formation in hypocotyls of *Eleutherococcus senticosus*. In the present study, the expression level of callose synthase gene in embryos of *E. senticosus* in response to 2,4-D, sucrose and mannitol treatments was analyzed by RT-PCR. The results show that plasmolysis pretreatment using sucrose and mannitol significantly promoted the expression of callose synthase gene. Also, the thicker cell walls of explant plasmolyzed compared with controls were observed during the somatic embryogenesis. We suggest that the callose may make the cells in epidermis separate from neighboring cells and then develop into embryogenic potential cells.

Keywords: *Eleutherococcus senticosus*; Somatic embryo; Plasmolysis; Callose synthase gene; RT-PCR

Introduction

Sexually generated embryos have provided plant breeders with an array of gene combinations for selection. Comparatively, the somatic embryos are regarded as a potentially stable, true-to-type source of plant propagation (Bonga and Durzan 1982). Somatic emrbyogenesis can be applied directly to mass clonal propagation of many normally seed propagated plants, including grains, vegetables, plantation crops, and forest trees (Hartmann et al. 1990). Growth regulators or other mechanical or chemical stress treatments play an important role in triggering somatic-embryo development (Kamada et al. 1989; Smith and Krikorian 1989; Choi and Soh 1997; Pasternak et al. 2002; Ikeda-Iwai et al. 2003). In general, 2,4- Dichlorophenoxyacetic acid (2,4-D) is an important inducer for somatic embryogenesis from zygotic embryos of some plant species, for example, *Eleuthercoccus senticosus* (Gui et al. 1991; Han and Choi 2003). Other stimulators or stressing agents acting on direct somatic embryogenesis, without phytohormonal inducers reported, such as high sucrose concentration or osmotic stressing agents (Kamada et al. 1993), heavy metal ions (Kiyosue et al. 1990; Pasternak et al. 2002), high temperature (Kamada et al. 1989) and plasmolysis (Choi and Soh 1997). The role of stress in somatic embryogenesis was reviewed by Karami and Saidi (2009).

Eleutherococcus senticosus (*Acanthopanax senticosus* also), an endangered medicinal plant, is a perennial deciduous shrub in Araliaceae family. According to Chinese medicine records, it has been used for 2 000 years to enhance the body's vital energy, restore memory, and prevent cold and flu. In Russia, *E. senticosus* was originally used by people in the Siberian Taiga region to increase performance and quality of life and to prevent infections. To improve the propagation of *E. senticosus via* embryogenic method will contribute to the resource protection of this plant. You et al. (2006) reported that the plasmolysis pretreatment could enhance the somatic embryogenesis in *E. senticosus* zygotic embryos in the absence of plant hormones and increase callose concentration. In the present study, we determined the effect of plasmolysis pretreatment on expression of callose synthase gene in *E. senticosus* zygotic embryo explants, and also observed the changes in cell wall after plasmolysis treatment.

Materials and methods

Seed stratification and culture of zygotic embryos

The seeds of *E. senticosus* were collected from Maoershan Mountain in Heilongjiang Provence, northeastern China. After

Foundation: This work was financially supported by the 948 Program of State Forestry Administration (No.2009-4-26), and co-sponsored by SRF for ROCS and the National Natural Science Foundation (No. 30972390) of China

The online version is available at http://www.springerlink.com

XILIN Hu • AN Yan • XIA De-an • YOU Xiang-ling (✉)

Key Laboratory of Forest Tree Genetic Improvement and Biotechnology (Northeast Forestry University), Ministry of Education of P. R. China, 26 Hexing Road, Harbin 150040, China. E-mail: yxiangling@yahoo.com
Tel: +86-451-82191752; Fax: +86-451-82191627

Responsible editor: Chai Ruihai

stratified in sand at 15°C for 6 months, the zygotic embryos inside the dehiscent seeds that developed into 4–6 mm-long cotyledon stage from globular stage were chosen. Seeds were sterilized, after coat removing, in 70% (v/v) ethyl alcohol for 1 min and 2% NaClO (w/v) for 20 min, and rinsed three times with sterile water. Zygotic embryos were dissected out from sterilized seeds, and cultured on half strength Murashige and Skoog (MS) medium (1962) with 2% (w/v) sucrose for 5 days to grow up to 1.5–2.0 cm in length.

Plasmolysis of explant and analysis of callose synthase gene (EsCS1) expression through RT-PCR

Choi et al. (1999) induced somatic embryos directly from the surface of hypocotyl segments of *Panax ginseng* cultured on MS medium with 4.5 μmol·L^{-1} 2,4-D. For 2,4-D pretreatment in present study, embryos were immersed in 4.5μmol·L^{-1} 2,4-D solution shaking for 2, 12 and 24 h, separately. For mannitol treatment, embryos were immersed in 1 mol·L^{-1} mannitol solutions shaking for 2, 12 and 24 h, separately. For sucrose treatment, embryos were immersed in 1 M reagent solutions shaking for 2, 12 and 24 h.

One putative callose synthase gene (EsCS1) fragment was identified from the expressed sequence tags in *E. senticosus* cDNA library, which is highly identical to callose synthase genes from *Arabidopsis thaliana* (81%; GenBank No. NM_111596) and *Vitis vinifera* (84%; GenBank No. AJ430780) (unpublished data). To study the EsCS1 expression profile, total RNA was prepared from plant samples using Trizol reagent kit (Invitrogen) and reverse transcribed with a RT-PCR kit (Takara, Japan). The primers (Forward: GAATTGGTACTTCGCAATTGCAGATC; Reverse: GCTTCATTGAAAAGGCGAGTTTGGA) specific to EsCS1 were used. RT-PCR analysis of ß-actin was used as control with a set of primers (Forward: TGGAAAA-GATTTGGCATCAC; Reverse: GACTGTGTGGCTGACA-CAT).

Histological observation

Embryos were pretreated in 1 mol·L^{-1} mannitol solution for 12 h, and the explants were cultured on MS medium containing 30 g/L sucrose and 3.0 g·L^{-1} Gelrite. The culture room was maintained at 23±2°C with a photoperiod of 16 h light (50 μmol·m^{-2}·s^{-1} under cool-white fluorescent tubes) and 8 h dark. Samples were fixed in 5% glutaraldehyde solution with 0.05 mol·L^{-1} phosphate buffer under 4°C and post fixed with 2% (w/w) OsO4 for 2 h. The samples were dehydrated in ethyl alcohol series and then embedded in epoxy resins. Pale golden sections (70–150 nm) were longitudinally cut from hypocotyls using LKB-V ultramicrotome and loaded onto 100-mesh copper grids coated with Formvar (1% in ethylene dichloride). Then these sections were stained with uranyl acetate and lead citrate. They were observed under transmission electron microscope (TEM) (Olympus CX31; Olympus, Tokyo, Japan) and images photographed with a digital camera (Nikon coolpix 995; Nikon, Tokyo, Japan).

Results

Expression of EsCS1 in response to plasmolysis and 2,4-D treatment

The expression of gene EsCS1 in response to various treatments was detected. There was no significant different expression of EsCS1 gene between in the hypocotyls after 2,4-D treatment and control (Fig. 1a), indicating that the treatment of 2,4-D in this experiment does not significantly enhance the EsCS1 expression. However, when the explant was plasmolyzed using 1 mol·L^{-1} mannitol or sucrose for 2 h or 12 h, the expression of EsCS1 was dramatically enhanced, whereas, EsCS1 expression decreased at 24 h of plasmolysis treatment (Fig. 1b, c). We supposed that plasmolysis treatment is an efficient inducer of EsCS1 expression; however, osmosis damage resulting from plasmolysis treatment for 24 h inhibited the EsCS1 expression in present study.

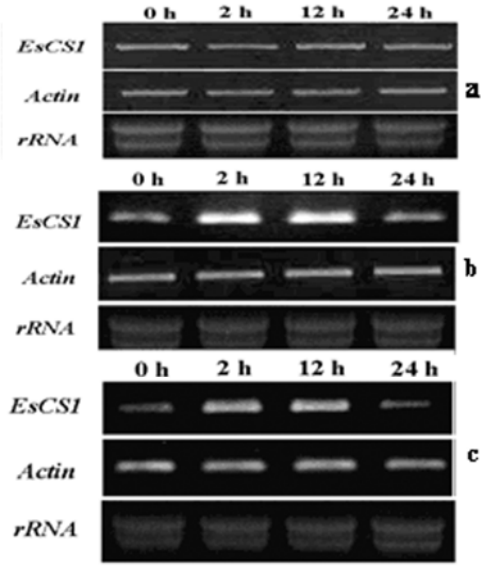

Fig. 1 Expression of callose synthase gene (EsCS1) in response to 2,4-D, mannitol and sucrose treatment by RT-PCR

Embryos were immersed in 4.5 μmol·L^{-1} 2,4-D, 1 mol·L^{-1} mannitol and 1 mol·L^{-1} sucrose solutions with shaking for 2 h, 12 h and 24 h, respectively. Actin was used as control for RNA integrity and accuracy of loading. a, total RNA was isolated from embryos with 4.5 μM 2,4-D treatment. 0 h, 2 h, 12 h and 24 h: 2,4-D treatment for 0 h, 2 h, 12 h and 24 h, respectively. b, Total RNA was isolated from embryos with 1 mol·L^{-1} mannitol treatment. 0 h, 2 h, 12 h and 24 h: 2,4-D treatment for 0 h, 2 h, 12 h and 24 h, respectively. c, total RNA was isolated from embryos with 1 mol·L^{-1} sucrose treatment. 0 h, 2 h, 12 h and 24 h: 2,4-D treatment for 0 h, 2 h, 12 h and 24 h, respectively.

Histological studies of somatic embryos obtained from pretreated explants

In order to reveal the effect of plasmolysis on somatic embryogenesis, the inter changes of sub-and epidermal cells through

histological section were observed. The ultrastructure observations showed that there was no significant difference in cell walls in un- and pre-treated zygotic embryos before culture on medium (Fig. 2a, b). After 4 weeks of culture, the cell walls of epidermal cell in pretreated explants became thicker than those in unpretreated (Fig. 2c, d), which made the intercellular space disappear. Also, at this time, a few plasmodesma was found in epidermal cell walls of unpretreated explants (Fig. 2e), but almost no plasmodesma of epidermal cell walls in pretreated explants was observed (Fig. 2f).

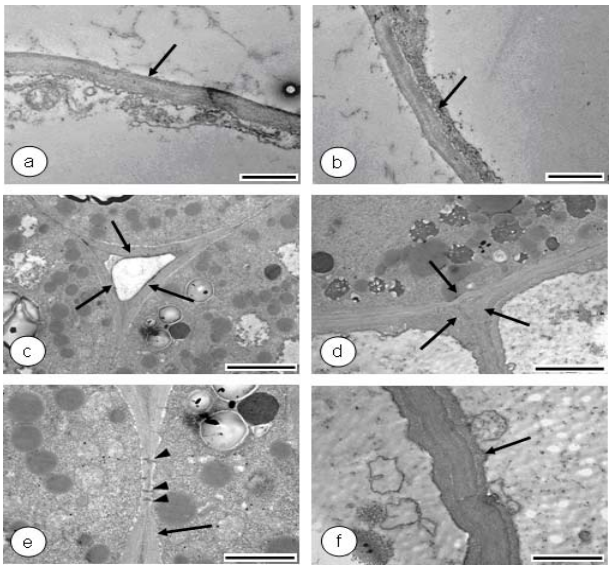

Fig. 2 Ultrastructure observation of cell walls in epidermis of explants during somatic embryogenesis (a, the cell wall of epidermis in unpretreated explant; b, the cell wall of epidermis in explant pretreated for 12-hour with mannitol; c, intercellular space between cells of epidermis in unpretreated explant after 4 weeks of culture; d, disappearing intercellular space between cells of epidermis in pretreated explants with mannitol after 4 weeks of culture; e, close-up views of the areas outlined in Fig.2c, arrow indicates the cell wall of epidermis in unpretreated explant, arrow heads indicate plasmodesmas; f: close-up views of the areas outlined in Fig. 2d, the cell wall of epidermis in pretreated explant. Bars: a and b, 500 nm; c and d, 2000nm; e and f, 1000μm)

Discussion

Callose [(1-3)-β-D-glucan] is synthesized by plants at many locations throughout development and in response to biotic and abiotic stress (Verma and Hong 2001). In most plant cells, callose is deposited only under conditions of stress, wounding, or pathogenesis (Ohana et al 1993). Previously, we have reported the callose accumulation after plasmolysis treatment (You et al. 2006). Consistent with the previous result, plasmolysis treatment could induce EsCS1 expression. In general, 2,4-D was a very important inducer of somatic embryogenesis in many other plants (Gui et al. 1991; Han and Choi 2003), as well in *E. senticosus*. You et al. (2006) reported that after three weeks of culture on MS medium containing 2,4-D, obvious callose signals around cell walls was found in proembryo-like structures formed on the surfaces of hypocotyls. However, 2,4-D treatment did not enhance the EsCS1 expression in the present study. Compared with plasmolysis treatment, 2,4-D treatment took more time of culture (more than three weeks) to induce somatic embryo. Therefore, it was a slow process for callose accumulation involved in 2,4-D treatment, and significant increase of EsCS1 expression couldn't be detected within 24 hours. Also, higher frequency of abnormally developed somatic embryos from hypocotyl segments treated by 2,4-D was found, however, numerous normal and singular somatic embryos formed directly on the surfaces of hypocotyls pretreated by plasmolysis, suggesting the different manners in embryo induction between 2,4-D treatment and plasmolysis treatment.

Wu and Cahoon (1995) demonstrated that plasmolysis facilitated the accumulation of protein and DNA into extra-plasmalemma spaces. In direct somatic embryogenesis from *Cichorium intybus* × *C. endivia* root segments, the first visible modification of the embryogenic cells is the deposition of a callosic wall, even without plasmolysis treatment (Dubois et al. 1990). In previous study, obvious callose accumulation was found in hypocotyl cells plasmolyzed with 1.0 mol·L^{-1} mannitol for 12 hours (You et al. 2006). In present study, that the thicker cell wall in hypocotyl cells plasmolyzed compared with control was checked only after several weeks of culture, suggesting the thickening of cell wall is a slow physiological process after the callose accumulation induced by plasmolysis treatment.

Grimault et al. (2007) indicated that embryogenic cell in leaves of Cichorium hybrid were surrounded by a cell wall that was characterized by the presence of callose. Choi et al. (1999) considered that interruption of cell-cell interaction by pretreatment might be the reason of enhancing single somatic embryo formation over the entire cotyledons preplasmolyzed, and callose accumulation in embryogenic cells might be related to embryogenic competency. From our results, we suggest that the suitable plasmolysis treatment enhances the expression of callose synthase gene and callose accumulation, sequentially result in thickening of cell wall of epidermal cell in pretreated explants, which makes the cells separate from neighboring cells and then develop into embryogenic potential cells.

References

Bonga JM, Durzan DJ. 1982. Tissue culture in forestry. The Hague: Martinus Nijhoff/DR W. Junk publishers, 125 pp.

Choi YE, Soh WY. 1997. Enhance somatic single embryo formation by plasmolyzing pretreatment from cultured ginseng cotyledons. *Plant Sci*, **135**: 197–206.

Choi YE, Yang DC, Yoon ES, Choi KT. 1999. High-efficiency plant production via direct somatic single embryogenesis from preplasmolysed cotyledons of Panax ginseng and possible dormancy of somatic embryos. *Plant Cell Rep*, **18**: 493–499.

Dubois T, Guedira M, Dubois J, Vasseur J. 1990. Direct somatic embryogenesis in roots of Cichorium: Is callose an early marker?. *Ann Bot*, **65**: 539–545.

Grimault V, Helleboid S, Vasseur J, Hilbert JL. 2007. Co-localization of β-1,3-glucanases and callose during somatic embryogenesis in Cichorium.

Plant Signal Behav, **2**: 455–461.

Gui Y, Guo Z, Ke S, Skirvin RM. 1991. Somatic embryogenesis and plant regeneration in Acanthopanax senticosus. *Plant Cell Rep*, **9**: 514–516.

Han JY, Choi YE. 2003. Mass production of Eleutherococcus seticosus plants through In vitro cell culture. *Korean J Plant Biotech*, **30**: 167–172.

Hartmann HT, Kester DE, Davies FT. 1990. Plant propagation: principles and practices. New Jersey: Prentice Hall Career & Technology Englewood Cliffs, 114 pp.

Ikeda-Iwai M, Umehara M, Shinobu S, Kamada H. 2003. Stress-induced somatic embryogenesis in vegetative tissues of Arabidopsis thaliana. *Plant J*, **34**: 107–114.

Kamada H, Ishikawa K, Saga H, Harada H. 1993. Induction of somatic embryogenesis in carrot by osmotic stress. *Plant Tiss Cult Lett*, **10**: 38–44.

Kamada H, Kobayashi K, Kiyosue T, Harada H. 1989. Stress induced somatic embryogenesis in carrot and its application to synthetic seed production. *In Vitro Cell Dev Biol*, **25**: 1163–1166.

Karami O, Saidi A. 2009. The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol Biol Rep DOI,10.1007/s11033-009-9764-3.

Kisyosue T, Takano K, Kamada H, Harada H. 1990. Induction of somatic embryogenesis in carrot by heavy metal ions. *Can J Bot*, **68**: 2301-2303.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, **15**: 473–497.

Ohana P, Benziman M, Delmer DP. 1993. Stimulation of callose synthesis in Vivo correlates with changes in intracellular distribution of the callose synthase activator -furfuryl-β-glucoside. *Plant Physiol*, **101**: 187–191.

Pasternak T, Prinsen E, Ayaydin F, Miskolezi P, Potters G, Asard H, Van Onekelen H, Dudits D, Fehér A. 2002. The role of auxin, pH and stress in the activation of embryogenic cell division in leaf protoplastderived cells of alfalfa (Medicago sativa L.). *Plant Physiol*, **129**: 1807–1819.

Smith DL, Krikorian AD. 1989. Release of somatic embryogenic potential from excised zygotic embryos of carrot and maintenance of proembryonic cultures in hormone-free medium. *Am J Bot*, **76**: 1832–1843.

Verma DPS, Hong Z. 2001. Plant callose synthase complexes. *Plant Mol Biol*, **47**: 693-701.

Wu FS, Cahoon AB. 1995. Plasmolysis facilitates the accumulation of protein and DNA into extra-plasmalemma space of intact plants cells. *Plant Sci*, **104**: 201-214.

You XL, Yi JS, Choi YE. 2006. Cellular change and callose accumulation in zygotic embryos of Eleutherococcus senticosus caused by plasmolyzing pretreatment result in high frequency of single-cell-derived somatic embryogenesis. *Protoplasma*, **227**: 105–112.